Laboratory Research

Effects of glucose and aldosterone on the proliferation of cardiac fibroblasts

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Objective To investigate the effects of glucose and aldosterone on the proliferation of rat cardiac fibroblasts. Methods The neonatal SD rat cardiac fibroblasts (Cfs) were separated by the differential attachment technique. Cfs were incubated in different D-glucose concentrations for 24 hours, with or without aldosterone. DNA synthesis and metabolic activity of the Cfs were measured simultaneously with Brdu incorporation and WST-1 ELISA, respectively. Results Glucose at high concentrations (15 and 25 mmol/L) significantly increased the proliferation of Cfs, compared with glucose at low concentration (5.6 mmol/L, P<0.01), while no difference was shown on Cfs proliferation between the two high glucose concentration groups. Addition of aldosterone (10^-7 mmol/L) to low-glucose media resulted in significant proliferation compared with those without aldosterone (WST-1, P<0.01; Brdu, P=0.019). However, when incubated in high glucose media, the stimulation effect of aldosterone for Cfs proliferation disappeared (P>0.05). Further analysis suggested that aldosterone have significant interaction on Cfs DNA synthesis (P=0.012). Conclusions Both glucose and aldosterone could stimulate the proliferation of cultured Cfs. In high glucose concentration, the stimulatory effect for Cfs proliferation may be masked.

Key words aldosterone; glucose; fibroblasts; cardiac; proliferation

Introduction

In recent years, several studies have indicated that local aldosterone was produced in the myocardium of normal rats and it had special effects on the heart. 1, 2 Aldosterone, mainly through nongenomic actions resulted in proliferation, fibrosis, inflammation, and tissue remodeling, 3 and there was cross-talk between its classic mineralocorticoid receptor (MR)-mediated effects and its non-genomic actions. 4 Glucose at high concentrations(10-30mmol/L)could stimulate cell proliferation in rat and human cardiac fibroblasts in vitro. 5 Hyperglycemia promoted cardiac fibrosis by different mechanisms, which included stimulating fibroblast proliferation and increasing interstitial accumulation of extracellular matrix temporarily or persistently. However, there were controversial results in whether high glucose and aldosterone had synergistic stimulatory effects on the proliferation of human cardiac fibroblasts. 5, 6 We therefore aimed to investigate the influence of glucose at different concentrations and aldosterone on the proliferation of neonatal rat cardiac fibroblasts and provide a new understanding of the occurrence of cardiac fibrosis.

Materials

Neonatal 1- to 3-day-old Sprague-Dawley rats were obtained from the Animal Centre of Hebei Medical University. Trypsin, Aldosterone, Spironolactone and L-glucose (U.S. sigma). Low-glucose DMEM culture medium, DMEM/F12 culture medium (Gibco). D-glucose (AMRESCO). Bovine serum albumin (BSA) (LANZHOU NATIONAL HYCLONE BIO-ENGINEERING CO.,LTD). WST-1 and Brdu kits (Boehringer Mannheim, Germany).ABC kit(Wuhan Boster Biological Technology,LTD).

Cell culture and identification

Neonatal 1- to 3-day-old Sprague-Dawley rats were killed and the hearts were removed quickly under sterile conditions. Cardiac fibroblasts (Cfs) were separated by enzymatic digestion and the differential attachment technique. 7 Then the cells were incubated at 37°C in humidified air with 5% CO₂. Complete medium was replaced every 2 days. When the cells grew to subconfluency, they were routinely split at a 1 : 2 ratio. Cfs at passage 2 were used for our study and were identified with light microscopy and SABC immunocytochemistry. 8

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Experimental cells were randomly divided into nine groups: (1) Low glucose group (5.6 mmol/L D-glucose); (2) Low glucose+10⁻⁷ mol/L Ald; (3) Low glucose+10⁻⁷ mol/L Ald+10⁻⁶ mol/L Spi; (4) High glucose group A (15 mmol/L D-glucose); (5) High glucose group A+10⁻⁷ mol/L Ald; (6) High glucose group A+10⁻⁷ mol/L Ald+10⁻⁶ mol/L Spi; (7) High glucose group B (25 mmol/L D-glucose); (8) High glucose group B+10⁻⁷ mol/L Ald; and (9) High glucose group A+10⁻⁷ mol/L Ald+10⁻⁶ mol/L Spi. The culture medium in each group were regulated to the same osmotic pressure using L-glucose.

Proliferation assay

DNA synthesis and metabolic activity of the Cfs were measured simultaneously with Brdu incorporation and WST-1 ELISA, respectively. In brief, after digestion, collection and counting, 5000-6000 cells/well were incubated in 96-well plate at the second passage. After incubated at 37 °C in humidified air with 5% CO₂ for 24 hours, the culture medium were replaced with Serum-free Low-glucose DMEM medium for another 24 hours to synchronize the cells. According to the experimental groups, the Cfs were treated by different factors (100 μl/well) for 24 hours, and then OD values were measured according to the manufacturer’s protocol.

Statistical analysis

All data were expressed as mean±SEM and analyzed by using statistics software SPSS13.0. Multiple groups means were compared with single factor analysis of variance, and the comparison among groups was performed with LSD method. Interaction was performed by two-way ANOVA. Values of *P*<0.05 were considered statistically significant.

Results

Proliferation of Cfs stimulated by D-glucose

Glucose at high concentrations (15, 25 mmol/L) significantly increased the proliferation of Cfs (WST-1, Brdu, *P*<0.01) compared with glucose at low concentration (5.6 mmol/L), while the effect of glucose on Cfs proliferation showed no difference between the two high glucose concentration groups. (Table 1, Fig.1)

Table 1 Effect of different D-glucose concentrations on proliferation of cultured neonatal rat cardiac fibroblasts (x±s, n=6)

<table>
<thead>
<tr>
<th>Group</th>
<th>WST-1</th>
<th>Brdu</th>
</tr>
</thead>
<tbody>
<tr>
<td>5.6 mmol/L Glu</td>
<td>0.270±0.016</td>
<td>0.206±0.018</td>
</tr>
<tr>
<td>15 mmol/L Glu</td>
<td>0.385±0.028 *</td>
<td>0.270±0.029 *</td>
</tr>
<tr>
<td>25 mmol/L Glu</td>
<td>0.417±0.034 *</td>
<td>0.279±0.045 *</td>
</tr>
</tbody>
</table>

* P<0.01 vs 5.6 mmol/L Glu, * P<0.05 vs 15 mmol/L Glu.

Proliferation of Cfs stimulated by aldosterone at different D-glucose concentrations

Addition of aldosterone to low-glucose DMEM resulted in significant (WST-1, *P*<0.01; Brdu, *P*=0.019) proliferation compared with those without aldosterone. However, when incubated in high glucose media, the stimulative effect of aldosterone for Cfs proliferation was lost (*P*>0.05). (Table 2, Fig.2)

Effects of spironolactone on the proliferation of Cfs

In three different glucose concentrations, 10⁻⁶ mmol/L spironolactone significantly (*P*<0.05) inhibited aldosterone-induced metabolic activity measured by WST-1, but spirono-
lactone did not show inhibitive effect on the DNA synthesis of the CFs induced by aldosterone (Table 3, Fig.3).

The interaction of D-glucose and aldosterone on the proliferation of CFs

Glucose with different concentrations and aldosterone had no significant interaction on the CFs metabolic activity \( (P=0.085) \), while they did have significant interaction on DNA synthesis \( (P=0.012) \).

![Image](image-url)

**Table 3** Effect of Spi on proliferation of cultured neonatal rat cardiac fibroblasts in different glucose concentrations \( (\bar{x} \pm s, n=6) \)

<table>
<thead>
<tr>
<th>Group</th>
<th>WST-1</th>
<th>Brdu</th>
</tr>
</thead>
<tbody>
<tr>
<td>5.6 mmol/L Glu+10^{-7} mol/L Ald</td>
<td>0.317±0.023</td>
<td>0.235±0.018</td>
</tr>
<tr>
<td>5.6 mmol/L Glu+10^{-7} mol/L Ald+10^{-6} mol/L Spi</td>
<td>0.265±0.041</td>
<td>0.205±0.020</td>
</tr>
<tr>
<td>15 mmol/L Glu+10^{-7} mol/L Ald</td>
<td>0.405±0.027</td>
<td>0.240±0.032</td>
</tr>
<tr>
<td>15 mmol/L Glu+10^{-7} mol/L Ald+10^{-6} mol/L Spi</td>
<td>0.361±0.015*</td>
<td>0.238±0.024</td>
</tr>
<tr>
<td>25 mmol/L Glu+10^{-7} mol/L Ald</td>
<td>0.415±0.025</td>
<td>0.240±0.017</td>
</tr>
<tr>
<td>25 mmol/L Glu+10^{-7} mol/L Ald+10^{-6} mol/L Spi</td>
<td>0.384±0.015*</td>
<td>0.243±0.015</td>
</tr>
</tbody>
</table>

**Discussion**

It is well-known that cardiac fibroblasts are important in producing and maintaining the extracellular matrix (ECM) of the heart. The abnormal proliferation of cardiac fibroblasts and deposition of the ECM protein, collagen, might adversely affect the performance of the heart.\(^{10}\) Hyperglycemia could work through both metabolic and hemodynamic pathways to change growth factors and ECM turnover. For example, hyperglycemia was responsible for the presence of high levels of nonenzymatically produced advanced glycation end-products (AGEs) in patients with diabetes, which were able to stimulate directly the production of ECM. And myocardial interstitial fibrosis might be present even in mild hyperglycemia in diabetes.\(^{11}\) The Renin-angiotensin-aldosterone system (RAAS) played an important role in the pathogenesis of diabetes. Aldosterone induced insulin resistance by inhibiting the biosynthesis and affinity of the insulin receptor (IR). It also down-regulated glucose transporters and increased fibrosis\(^{12}\) and there was abundant evidence linking aldosterone, through non-genomic actions, to defective intracellular insulin signaling and impaired glucose homeostasis.\(^{3}\) Current data suggest that cardiac fibroblasts contain a functional intracellular RAS which participates in extracellular matrix formation in high glucose conditions.\(^{13}\) Therefore, the interaction of aldosterone and glucose played an important role in the development of cardiac fibrosis in patients with diabetes.

In this experiment, in order to exclude the impact of osmotic pressure, we used L-glucose, which could not be metabolized by cells, to equalize osmotic pressure of culture medium with different glucose concentrations. In this case, the proliferation of CFs were still significantly stimulated by high glucose, showing that regardless of whether the osmotic pressure was involved in cell proliferation, the effect of high glucose on the proliferation was without doubt. The biochemical mechanisms were proposed including activation of protein kinase C (PKC), extracellular signal-regulated kinase (ERK) pathway, and P38 mitogen-activated protein kinase (MAPK) pathway.\(^{14}\)

The literature concerning the influence of aldosterone on development of cardiac fibrosis was controversial. In vivo experiments in rat models of hyperaldosteronemia showed a pro-fibrogenic effect of aldosterone, whereas in vitro data of rat cultured cardiac fibroblasts provided conflicting results.\(^{8, 15}\) Some experiments showed that part of the aldosterone induced effects were mediated through crosstalks, which meant that a primary non-genomic effect resulted in a DNA-mediated change of protein expression.\(^{16}\) And in the case of non-genomic effect of aldosterone, a regulatory effect of proteins that were connected with structural proteins, signal cascades, osmoregulation and calcium pathway as well as the general metabolism was discovered.\(^{4}\)

In our experiment, WST-1 and Brdu revealed that under the surroundings of low glucose, aldosterone significantly stimulated the DNA synthesis and metabolic activity of the CFs and the stimulative effects could be inhibited by spironolactone, showing the stimulative role of aldosterone by classical receptor pathway. This also supported using of antagonists of the MR as a part of a commonly applied standard therapy in treating heart disease.

We also found high glucose and aldosterone had interaction on DNA synthesis. For cardiomyocytes, there were strong evidence that the effect of high glucose on aldosterone-induced hypertrophy was dependent on the capacity...
of glucose to activate PKC. That meant D-glucose and aldosterone may have the same pathways of promoting cell proliferation or the stimulative effects of aldosterone might be masked under stronger role by high glucose. Although the relationship among aldosterone, glucose metabolism and insulin resistance was not well understood. In the year 2000, the Expert Committee on the Diagnosis and Classification of Diabetes Mellitus suggested that primary aldosteronism was a unique form of diabetes. We still need more studies on human cardiac fibroblasts and further in vivo.

In summary, aldosterone stimulates the proliferation of fibroblasts through the MR and non-genomic pathways, and mechanisms of the interaction with high glucose need to be further studied.

References